IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Kelichi FUKUDA et al.	(Conf.:	9902
Appl. No.:	10/574,530	(Group:	1632
Filed:	October 4, 2004	(Examiner:	Valarie Bertoglio
For:	METHOD OF INDUCING THE DIFFERENTIATION OF STEM CELLS INTO MYOCARDIAL CELLS		

DECLARATION UNDER CFR 1.132

Honorable Commissioner for Patent

P.O. Box 1450

Alexandria, VA 22313-1450

Sir:

I, Uichi KOSHIMIZU, declare and state that I am one of the applicants of the above-identified patent application.

I declare that I graduated in March 1988 from the Faculty of Agriculture, Meiji University, Kanagawa, Japan, and that I received a bachelor's degree in Agriculture from the same University. I further declare that I graduated in March 1994 from Osaka University Medical School, Osaka, Japan, and that I received Ph.D. in Medicine from the same University.

I declare that I was employed as an assistant professor and lecturer by Osaka University Medical School during 1995-2001, and that I have been employed as a senior researcher by SUNTORY Biomedical Research Co., Ltd. (renamed as ASUBIO Pharma Co., Ltd.) since 2001. I have been engaged as a manager and a chief researcher of Medical Biotechnology Group of ASUBIO Pharma since 2007.

I declare further that I have read all of the Official Actions in the above-identified application, and have read and am familiar with each of the references cited in the Official Action by the Examiner.

I declare further that the following tests were conducted at my direction or under my supervision, and that the test results are true and correct to the best of my knowledge.

I declare further that all statements made herein are of my own knowledge and true, and that all statements made on the basis of information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated this *12th* day of January, 2010

By: Uichi Koshimizu

Uichi KOSHIMIZU

EXPERIMENT REPORT

January 12, 2010

By Uichi KOSHIMIZU,
Manager and Chief Researcher
of ASUBIO Pharma Co., Ltd,
one of the inventors of the present application

The specification of U.S. Patent Application No. 10/574,530 (hereinafter referred to as the present application) shows in Examples that pluripotent stem cells were differentiated into cardiomyocytes using a substance that inhibits BMP signaling, which includes BMP antagonists such as Noggin and Chordin. The results shown in the Examples of the specification suggests that cardiomyocytes can be differentiated from pluripotent stem cells of primates, such as monkey and human, by inhibiting function and signaling of an endogenous BMP molecule.

Experiment 1: Effects of BMP4-siRNA Treatment on Cardiomyocyte-Differentiation from ES Cells

BMP4 is a molecule of BMP family, and expressed in undifferentiated and early stages of differentiation. In this experiment, we confirmed that introduction and expression of siRNA specific to BMP4 efficiently induced cardiomyogenic differentiation from ES cells.

According to the same methods as in Example 1 of the present specification, murine ES cells were passaged and maintained in an undifferentiated state, and 3 days before the starting of differentiation induction, the ES cells were dispersed into single cells by trypsin treatment. Then, siRNA specific to BMP4 gene (BMP4-siRNA; available from Ambion) was introduced into the cells using Nucleofector (available from Amaxa), and expressed. The sequences of the BMP4-siRNA were as follows.

(forward) 5'-CCACAGCGGUCCAGGAAGATT-3'

(reverse) 5'-UCUUCCUGGACCGCUGUGGTT-3'

Expression of the introduced BMP4-siRNA in the cells continued for 72 hours and more, and gene expression of BMP4 was clearly inhibited. After culturing the cells for 3 days in undifferentiated state, EBs were formed by the same methods as in Example 6 using floating culture plates (SUMILONCELLTIGHT Multiwell Plates; available from Sumitomo Bakelite) to induce differentiation. In the differentiation induction stage, the ES cells were incubated with the medium-containing siRNA for 1 day, and the effects of treated-siRNA, i.e. the decrease of BMP4 expression level by BMP4-siRNA, was confirmed to remain at least for the first 3 days.

As shown in **Figure 1**, very few of the EBs in the non-treated group ("none") exhibited beating even after 14 days of culture. On the other hand, in the groups treated with BMP antagonists such as Chordin and DAN, beating was confirmed in about 50-60% of EBs on the 10^{th} day of differentiation. Autonomous beating was also confirmed in about 40% of EBs in the group treated with BMP4-siRNA. Further, in the non-treated group, beating was limited to certain restricted regions of the EBs, while in the groups treated with BMP antagonists and BMP4-siRNA, beating was confirmed virtually throughout almost regions of EB surface.

Next, expression of cardiomyocyte-specific marker genes was investigated using the same methods as in Example 2 of the present specification. As shown in **Figure 2**, strong expression of the GATA4, Nkx2.5, α MHC, βMHC and other genes was confirmed on the 14^{th} day of differentiation in the groups treated with Chordin, DAN, or BMP4-siRNA, compared to that in the non-treated group.

Protein production of cardiomyocyte-specific markers was then investigated by immunocytochemical staining using the same methods as in Example 2 of the present specification. The results are shown in **Figure 3**. While cells positive for cardiomyocyte-specific markers were observed only in very limited regions of the non-treated group EBs, most of the cells comprised in the EBs showed positive and strong signals in the group treated with

BMP4-siRNA as well as in the groups treated with BMP antagonists (Chordin and DAN).

These results show that beating cardiomyocytes appeared from ES cells in which gene expression of BMP4 was inhibited by introduction and expression of BMP4-siRNA, as much as from ES cells treated with BMP antagonists.

This experiment demonstrates the effect of BMP4-siRNA treatment on cardiomyocyte-differentiation from ES cells, of which BMP4 mRNA expression level was decreased within the first 3 days of the differentiation-inducing stage.

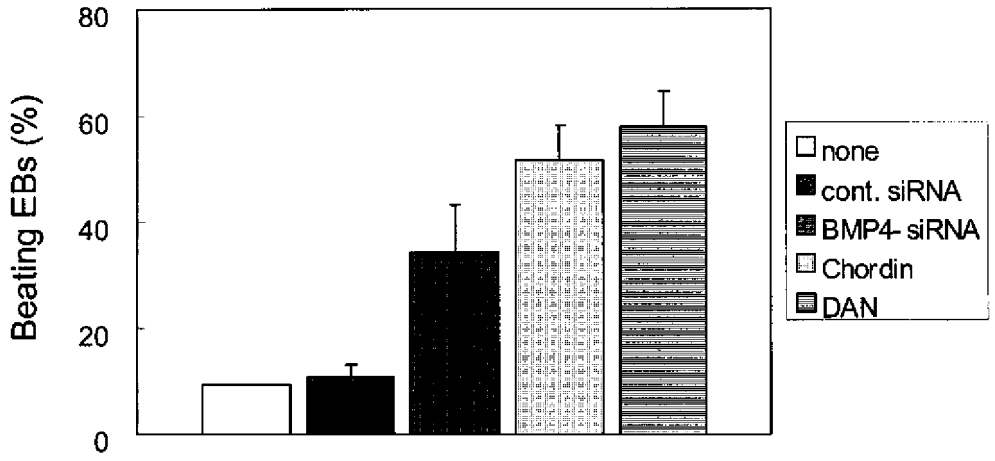

Figure 1. Effects of BMP4-siRNA on appearance rate of beating EBs from mouse ES cells. The appearance rate of beating EBs was measured on the 10^{th} day after inducing differentiation (n=3).

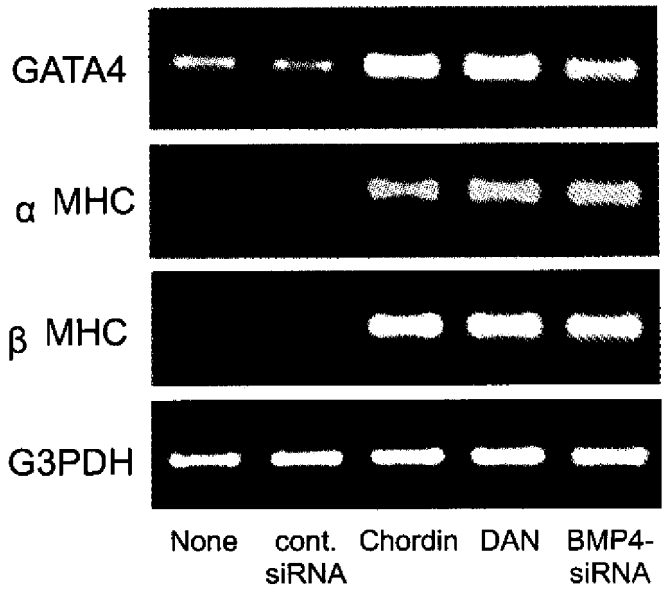

Figure 2. Effects of BMP4-siRNA on expression of cardiomyocytes marker genes. EBs, which were differentiated from ES cells under the same conditions as in Figure 1, were collected 6 days after EB formation, and the expression of each gene was analyzed.

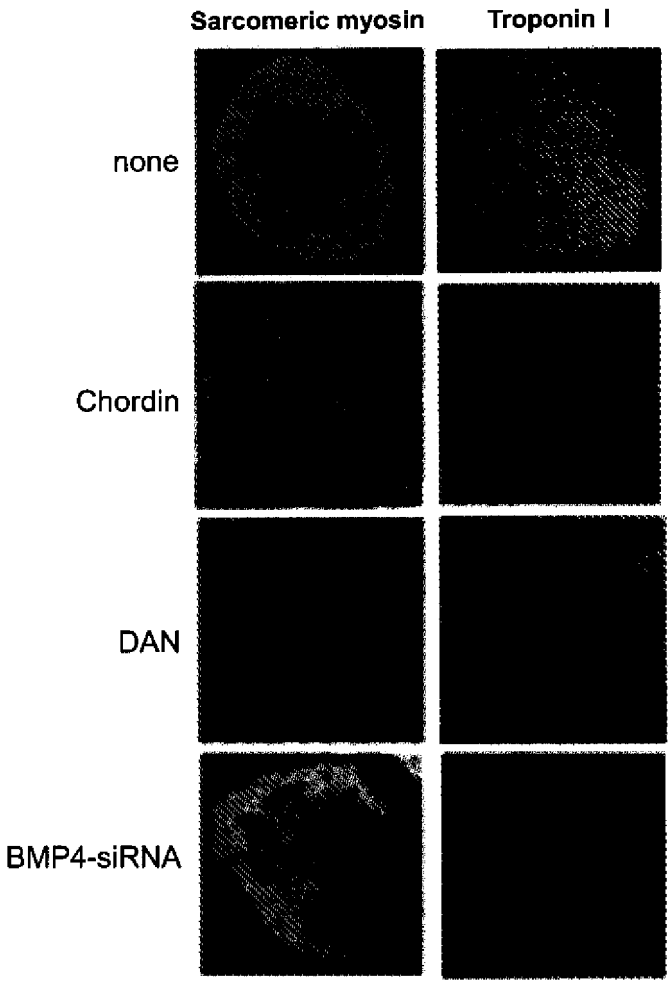

Figure 3. Effects of BMP4-siRNA on expression of cardiomyocytes-specific marker proteins. EBs, which were differentiated from ES cells under the same conditions as in Figure 1, were collected 14 days after EB formation, and were stained with each antibody against indicated protein.

Experiment 2: Effects of BMP Antagonist Treatment on Cardiomyocyte-Differentiation from Common Marmoset ES Cells

The monkey, common marmoset ES (cm-ES) cell lines No. 20, 30 and 40 (Sasaki et al., Stem Cells 23: 1304, 2005) were obtained from the Laboratory of Applied Developmental Biology, Marmoset Research Department, Central Institute for Experimental Animals, Japan.

cm-ES cells were cultured on 10 μg/mL mitomycin C-treated mouse embryonic fibroblast (MEF) feeder cells with culture medium, which consisted of 80% Knockout Dulbecco's modified Eagle's medium (KO-DMEM; Invitrogen Co., 10829-018) supplemented with 20% Knockout Serum Replacement® (KSR; Invitrogen Co., 10828-028), 0.1 mM MEM Non-Essential Amino Acids Solution (Sigma-Aldrich Co., M7145), 2 mM L-Glutamine (Invitrogen Co., 25030-081), 0.1 mM β-Mercaptoethanol (2-ME; Sigma-Aldrich Co., M-7522) and 4 ng/mL basic fibroblast growth factor (bFGF; Wako Pure Chemical Industries Ltd., 064-04541), designated thereafter as cm-ES cell medium, CMESM. cm-ES cells were passaged every 5 or 6 days to maintain them in an undifferentiated state.

The cm-ES cells cultured for 3 days in CMESM treated with a recombinant protein of Noggin, Chordin, or DAN (50 ng/mL; available from R&D Systems) or in non-treated CMESM, and used in the following procedures. Colonies of cm-ES cells were dissociated from the medium using a primate ES cell dissociation solution (available from ReproCell), and colonies having a size of 40-100 μm were prepared for a later differentiation induction. The aggregates of the cm-ES cells were suspended in a culture medium, to which was added the above each recombinant protein after removing bFGF from CMESM, to induce differentiation using floating culture plates (available from Valmalk). In the differentiation induction stage, the cm-ES cells were treated with Noggin or Chordin for the first 3 days.

As shown in **Figure 4**, very few of the EBs in the non-treated group ("none") exhibited beating even after 14 days of culture. On the other hand, in the groups treated with Noggin, Chordin or DAN, beating was observed beginning on the 10^{th} day of differentiation, and beating was confirmed in significantly high percentages of EBs on the 14^{th} day compared to that in the non-treated group.

Next, gene expression and protein production of various cardiomyocyte-specific markers were investigated using the same methods as in Example 2 of the present specification.

Expression of various cardiomyocyte-specific marker genes in the Noggin-treated group, the

DAN-treated group and the non-treated group is shown in **Figure 5**. The primers used to detect the transcripts of common marmoset ANP (atrial natriuretic peptide), MLC2a (myosin light chain-2a), MLC2v (myosin light chain-2v) and β-Actin were as follows.

ANP	(forward)	5'-GAACCAGAGGGGAGAGACAGA-3'
ANP	(reverse)	5'-CCCTCAGCTTGCTTTTTAGGAG-3'
MLC2a	(forward)	5'-GAGGAGAATGGCCAGCAGGAA-3'
MLC2a	(reverse)	5'-GCGAACATCTGCTCCACCTCA-3'
MLC2v	(forward)	5'-AGGAGGCCTTCACTATCATGG-3'
MLC2v	(reverse)	5'-GTGATGATGTGCACCAGGTTC-3'
β-Actin	(forward)	5'-TCCTGACCCTSAAGTACCCC-3'
β-Actin	(reverse)	5'-GTGGTGGTGAAGCTGTAGCC-3'

Expression of cardiomyocyte-specific genes such as ANP, MLC2a and MLC2v in EBs of the groups treated with Noggin and DAN was significantly greater than in EBs of the non-treated group.

While cells positive for cardiomyocyte-specific marker proteins such as α -Actinin, Nkx-2.5, GATA-4 and troponin I were observed only in very limited regions of the non-treated group EBs, in the group treated with BMP antagonists such as Noggin and DAN, cardiomyocyte marker-positive cells were found in a wide range of the EBs (**Figure 6**).

These results show that the present invention certainly promoted differentiation of cardiomyocytes from monkey (common marmoset) ES cells.

This experiment demonstrates the effect of BMP antagonist treatment on cardiomyocyte-differentiation from common marmoset ES cells when these ES cells are treated with a BMP antagonist within 3 days before inducing differentiation and within the first 3 days of the differentiation-inducing stage.

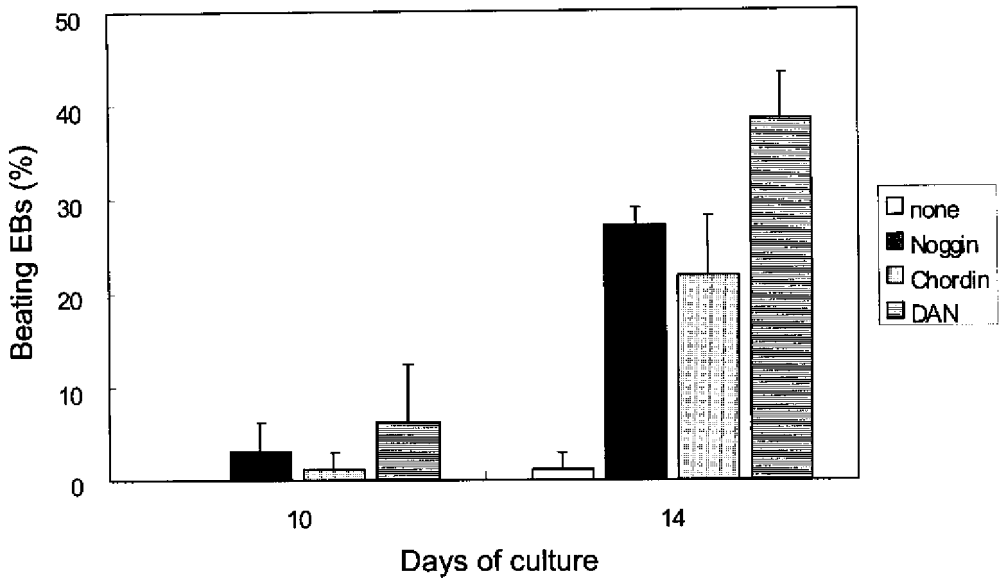

Figure 4. Effects of BMP antagonists on appearance rate of beating EBs from common marmoset ES cells. n=3.

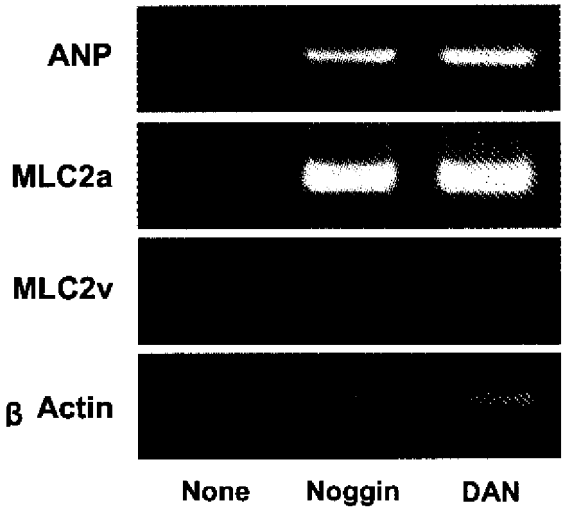

Figure 5. Effects of BMP antagonists on expression of cardiomyocytes-specific marker genes. EBs, which were differentiated from common marmoset ES cells, were collected 16 days after EB formation, and the expression of each gene was analyzed.

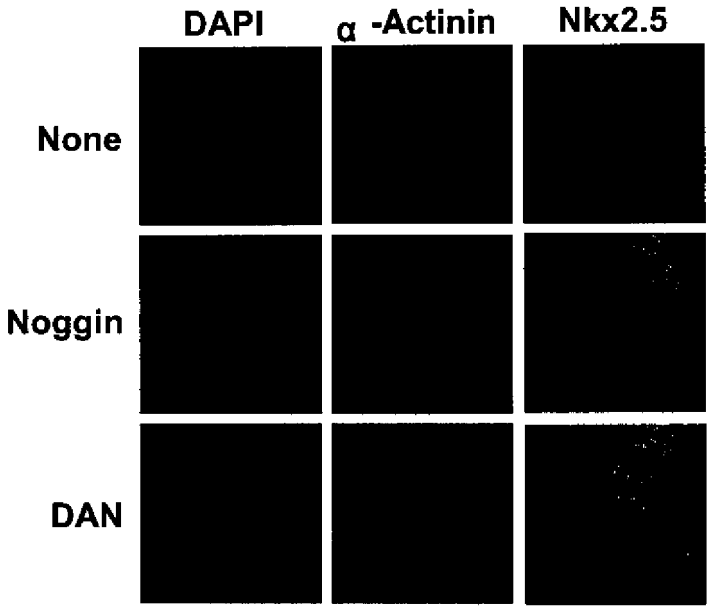

Figure 6. Effects of BMP antagonists on expression of cardiomyocytes-specific marker proteins. EBs, which were differentiated from common marmoset ES cells, were collected 16 days after EB formation, and were stained with each antibody against indicated protein. DAPI: 4', 6-diamino-2-phenylindole

Experiment 3: Effects of BMP Antagonist Treatment on Cardiomyocyte-Differentiation from human ES Cells and human iPS Cells

Human ES (h-ES) cell lines KhES-1, KhES-2 and KhES-3 were obtained from Kyoto University, Japan (Suemori et al., Biochem. Biophys. Res. Commun. 345: 926, 2006). Human iPS (h-iPS) cell lines 201B6, 201B7, 253G1, and 253G4 were obtainted from Kyoto University, Japan (Takahashi et al., Cell 131: 861, 2007; Nakagawa et al., Nat. Biotechnol. 26:101, 2008).

h-ES cells and h-iPS cells were cultured on 10 μg/mL mitomycin C-treated mouse embryonic fibroblast (MEF) feeder cells with culture medium, which consisted of 80% DMEM/F12 (Sigma-Aldrich) supplemented with 20% Knockout Serum Replacement® (KSR; Invitrogen), 1mM L-glutamine, 1mM MEM Non-Essential Amino Acids Solution (Sigma–Aldrich), 0.1mM β-mercaptoethanol (2-ME; Sigma–Aldrich) and 4ng/ml basic fibroblast growth factor (bFGF;

Wako Pure Chemical Industries Ltd.,) , designated thereafter as human pluripotent stem cell medium, HPSM. h-ES cells and h-iPS cells were passaged every 5 or 6 days to maintain them in an undifferentiated state.

The h-ES cells and h-iPS cells were cultured for 3 days in HPSM treated with a recombinant protein of Noggin or Chordin (50 ng/mL; available from R&D Systems) or in non-treated HPSM, and used in the following procedures. Colonies of h-ES cells and h-iPS cells were dissociated from the medium using a primate ES cell dissociation solution (available from ReproCell), and colonies having a size of 40-100 μm were prepared for a later differentiation induction. The aggregates of the h-ES cells and h-iPS cells were suspended in a culture medium, to which was added the above each recombinant protein after removing bFGF from HPSM, to induce differentiation using floating culture plates (available from Valmalk). In the differentiation induction stage, the h-ES cells and h-iPS cells were treated with Noggin or Chordin for the first 3 days.

Approximately 0.1-1% of the EBs derived from h-ES cells and h-iPS cells in the non-treated group exhibited beating on the 24^{th} day of floating culture. On the other hand, in 2-5% of EBs derived from h-ES cells and h-iPS cells treated with Noggin or Chordin, beating was observed on the 14^{th} day of floating culture, and beating was confirmed in 10-15% of EBs on the 24^{th} day.

To visualize the existence of cardiomyocytes in the beating EBs, staining by a fluorescent probe for mitochondria was conducted. In this regard, it was reported by one of the present inventors that, since cardiomyocytes contain more mitochondria and higher mitochondrial membrane potential than the other types of cells, cardiomyocytes can be isolated and purified by labeling using a probe specific to mitochondria in live cells and by monitoring membrane potential of the labeled mitochondria (WO2006/022377; Hattori F et al., Nature Methods, in revision). Accordingly, cardiomyocytes in EBs can be labeled and observed by fluorescence staining using the fluorescence probe for mitochondria.

Fluorescence probe for mitochondria (MitoTracker® Red: MTR; available from Molecular

Probe) was added in the culture in a final concentration of 100 nM, and EBs in the culture were incubated at 37°C for 10 minutes, and then observed using a fluorescence microscope.

In the non-treated group ("none"), very few of EBs derived from h-ES cells and h-iPS cells exhibited fluorescence from MTR label on the 24th day of floating culture (**Figure 7** and **Figure 8**). On the other hand, in the groups treated with Noggin and Chordin, EBs exhibiting fluorescence from MTR label were observed from around the 14th day of culture, and strong fluorescence from MTR label was confirmed in beating EBs. Further, on the 24th day of differentiation, strong fluorescence from MTR label was clearly observed in whole EBs, and most of the EBs had beating property.

This experiment demonstrates the effect of BMP antagonist treatment on cardiomyocyte-differentiation from h-ES cells and h-iPS cells when these cells are treated with a BMP antagonist within 3 days before inducing differentiation and within the first 3 days of the differentiation-inducing stage, and show that the present invention certainly promoted differentiation of cardiomyocytes from human pluripotent stem cells.

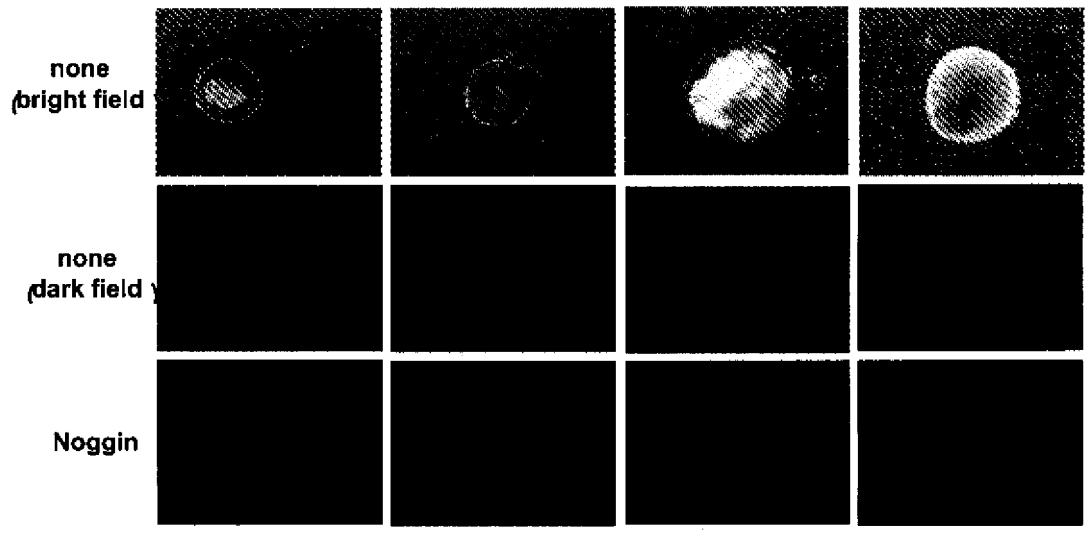

Figure 7. Effects of BMP antagonists on differentiation of cardiomyocytes from human ES cells. human ES cells (KhES3 cell line) were treated with Noggin, and fluorescent image

of EBs was obtained using MitoTracker® Red on the 24th day after inducing differentiation. Upper panels depict bright-field images of the untreated EBs. Middle panels show the corresponding fluorescence images. Lower panels depict fluorescent images of EBs treated with Noggin.

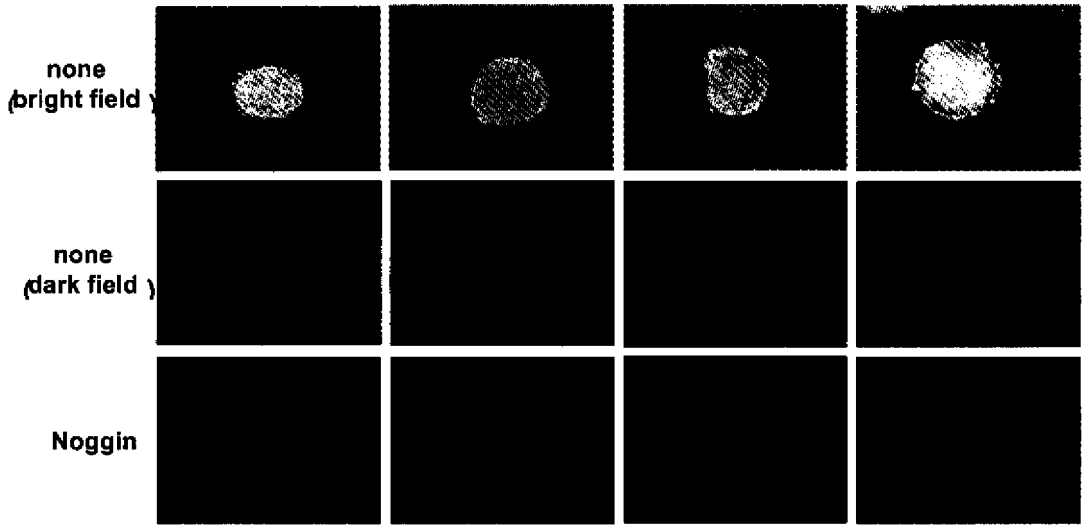

Figure 8. Effects of BMP antagonists on differentiation of cardiomyocytes from human iPS cells. human iPS cells (201B7 cell line) were treated with Noggin, and a fluorescent image of EBs was obtained using MitoTracker® Red on the 24th day after inducing differentiation. Upper panels depict bright-field images of the untreated EBs. Middle panels show the corresponding fluorescence images. Lower panels depict fluorescent images of EBs treated with Noggin.

In addition, I confirm that, in Example 6 in the specification of the present application, human embryonal carcinoma cells were treated with Noggin or Chordin for 3 days before inducing differentiation and for the first 3 days of differentiation-inducing stage. Therefore, the example demonstrates the effect of BMP antagonist treatment on cardiomyocyte-differentiation from human embryonal carcinoma cells when these cells are treated with a BMP antagonist within 3 days of pre-differentiation stage and within the first 3 days of the differentiation-inducing stage.

Exhibit A

Curriculum Vitae

January 4, 2010

Name: Uichi KOSHIMIZU
Degree: Ph.D. (Medicine)

Present Address: (Laboratory)
Asubio Pharma Co., Ltd.
Biomedical Research Laboratories
1-1-1 Wakayama-dai, Shimamoto-cho, Mishima-gun,
Osaka 618-8513, Japan
Tel: +81-75-962-8304
Fax: +81-75-962-6448
E-mail: koshimizu.uichi.ss@asubio.co.jp
(Home)
15-1 Hyakuyama, Shimamoto-cho, Mishima-gun,
Osaka 618-0021, Japan
Position: Manager and chief researcher of Medical Biotechnology Group

Date of Birth: June 21, 1965 (Age:44)
Nationality: Japanese

Education and Positions:

1984-1988 Faculty of Agriculture at Meiji University (Kanagawa, Japan)
Advisor: Prof. H. Nagasawa

1988-1994 Ph.D. candidate of Osaka University Medical School (Osaka, Japan)
Advisor: Prof. Y. Nishimune
(Research Insitute for Microbial Diseases)

1993-1995 Research fellow, National Institute of Genetics (Mishima, Japan)
Advisor: Prof. N. Nakatsuji

Received Ph.D. in March, 1994, from Osaka University Medical School

1995-2001	Assistant Professor & Lecturer, Osaka University Medical School (Osaka, Japan)
2001-2007	Senior Researcher, Suntory Biomedical Research Co. Ltd. (renamed as Asubio Pharma Co. Ltd)
2007-	Manager and chief researcher of Medical Biotechnology Group
Awards:	Fellowships of the Japan Society for the Promotion of Science for Junior Scientists (1992-1994, 1994- 1995)
Memberships:	Japanese Society of Developmental Biologists Molecular Biological Society of Japan Japanese Society of Regenerative Medicine Japan Society of Gene Therapy International Society for Stem Cell Research

Publication List

Articles (in English): 50
Reviews (in English): 1
Reviews (in Japanese): 30
Patent specifications: 4

The important articles

1. U. Koshimizu, D. Watanabe, Y. Tajima, and Y. Nishimune
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24. M. Nagaoka, H. Ise, I. Harada, U. Koshimizu, A. Maruyama, and T. Akaike
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Patent specifications

1. "Method of inducing the differentiation of stem cells into myocardial cells" (WO2005-033298)

2. "Method of growing myocardial cells" (WO2005-049822)

3. "Method of proliferating pluripotent cells" (WO2005-090557)

4. "Method of preparation of cardiomyocytes from pluripotent stem cells" (WO2007-126077)